Folia Neuropathologica

Clinical presentation of Y189C mutation of the *NOTCH3* gene in the Polish family with CADASIL

Jolanta Dorszewska[1*], Marta Kowalska[1*], Tomasz Grzegorski[2,3], Dorota Dziewulska[4,5], Katarzyna Karmelita-Katulska[6], Anna-Maria Barciszewska[7], Michał Prendecki[1], Wojciech Gorczyński[2], Wojciech Kozubski[2]

[1]Laboratory of Neurobiology, Department of Neurology, Poznan University of Medical Sciences, Poznan, Poland, [2]Chair and Department of Neurology, Poznan University of Medical Sciences, Poznan, Poland, [3]Department of Clinical Neuroimmunology, Poznan University of Medical Sciences, Poznan, Poland, [4]Department of Experimental and Clinical Neuropathology, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, [5]Department of Neurology, Medical University of Warsaw, Poland, [6]Department of General Radiology and Neuroradiology, Poznan University of Medical Sciences, Poznan, Poland, [7]Department and Clinic of Neurosurgery and Neurotraumatology, Poznan University of Medical Sciences, Poznan, Poland

*These authors contributed equally to this work.

Folia Neuropathol 2020; 58 (1): 83-92 *DOI: https://doi.org/10.5114/fn.2020.94009*

Abstract

Introduction: *Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary, progressive ischemic disease of small vessels of the brain characterized by migraine with aura (MA), recurrent subcortical ischemic episodes, cognitive decline and psychiatric disorders. CADASIL is caused by mutations in the NOTCH3 gene. We identified the NOTCH3 Y189C mutation as a genetic cause of CADASIL in a Polish family and provided its first clinical manifestation.*

Material and methods: *The study included twelve subjects from one family. The NOTCH3 mutation, APOE and MTHFR polymorphisms were determined by high-resolution melting analyses (HRMA) and Sanger sequencing. Neuroimaging included CT and MRI. Ultrastructural examination of skin-muscle biopsy material of the proband was performed.*

Results: *The NOTCH3 Y189C mutation was present in a 36-year-old woman and her two sisters (aged 40 and 27) from 6 siblings. The MA was found in all of them, and started or became more severe after childbirth. The numerous T2/FLAIR hyperintense lesions were shown in the brain MRI. The deposition of granular osmiophilic material in the wall of small vessels of the proband observed in histopathological analysis confirmed the high degree of CADASIL severity.*

Conclusions: *Patients with the Y189C mutation of NOTCH3 from the same family display a similar phenotype of CADASIL.*

Key words: *NOTCH3, Y189C mutation, CADASIL, clinical pattern.*

Introduction

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary, progressive disease of small cerebral arteries. CADASIL is characterized by migraine with aura (MA), recurrent subcortical ischemic events, vas-

Communicating author

Prof. Jolanta Dorszewska, Laboratory of Neurobiology, Department of Neurology, Poznan University of Medical Sciences, 49 Przybyszewskiego St., 60-355 Poznan, Poland, phone: +48 61 86 91 439, fax: + 48 61 86 91 697, e-mail: dorszewskaj@yahoo.com

cular dementia and psychiatric disorders. In 20-40% of patients, the first symptom is MA, often with atypical aura, started in the second-third decade of life. Ischemic events, typically presented as a lacunar syndrome, occur in about 85% of symptomatic patients at the mean age of 46. Ischemic episodes are preceded by changes in brain MRI which are found in almost all patients after the age of 35. Cognitive decline may be presented in 60% of symptomatic individuals by the age of 65 [3-5].

The disease is caused by a mutation in the *NOTCH3* gene which is localized on chromosome 19, consists of 33 exons and encodes 2321 amino acids transmembrane receptor NOTCH3. NOTCH3 is crucial for the development of vascular smooth muscle cells (VSMC) and their functioning. Moreover, NOTCH3 protects against apoptosis and regulates the response of VSMC to external factors. NOTCH3 receptor is expressed in VSMC and pericytes, mostly in the arteries and capillaries, and some subpopulations of thymocytes and lymphocytes T [6,9,17,19].

The extracellular part of NOTCH3 consists of 34 epidermal growth factor-like (EGF-like) repeats, each with six cysteine residues; three Lin-12/NOTCH repeat (LNR) protecting from ligand-independent activation of the receptor and the heterodimerisation domain (HD) being the site of cleavage. Whereas, the intracellular part of the protein is made up by seven ankyrin repeats (ANK) surrounded by Rbp-associated molecule (RAM) domain and peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST). The EGF-like repeats 10 and 11 are essential for ligand binding [25].

The genetic testing is a gold standard for confirming the diagnosis of CADASIL. More than 200 mutations in *NOTCH3* were identified, the most of them occur in exons 4 and 3 [24]. Majority of mutations are missense variants (~95%), but the small in-frame deletions and splice-site mutations also can be found. Typical mutations responsible for CADASIL are situated in the EGF domain, encoded by exons 2-24 of *NOTCH3,* and lead to a change of the cysteine residues from regular six into odd-number. These mutations change the three-dimensional structure of the protein, impair the interaction of NOTCH3 with itself and other proteins, and promote multimerization of NOTCH3 [16]. The amino acid substitutions not changing the cysteine residues were also observed. However, their significance to CADASIL is controversial [24].

The mutations in the *NOTCH3* gene result in the degenerative changes in brain arteries, including aggregation of the extracellular domain of the NOTCH3 protein, deposition of granular osmiophilic material (GOM) around the pericytes and VSMC, remodelling and declining functions of these cells. It is reflected in a decrease in cerebral blood flow and reduction in cerebral vasoreactivity, which in consequence leads to chronic ischemia. Vascular abnormalities are found not only in the brain, but also in other organs, e.g., heart, kidney, skin and muscles [9,16,30,34].

We identified the Y189C (p.Tyr189Cys, c.A642G) mutation in the *NOTCH3* gene and provided its first clinical manifestation in CADASIL.

Material and methods

The study was approved by the Local Bioethical Committee of the Poznan University of Medical Sciences (no. 931/14 with extension no. 993/17). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Our proband (II-6) and her sisters (II-2 and II-11) fulfil the International Classification of Headache Disorders, 3rd edition (beta version) criteria for MA [14] and present with clinical symptoms of CADASIL.

Genetic analyses

Genomic DNA was extracted from blood using isolation kit Genomic Micro AX Blood Gravity (A&A Biotechnology, Poland) and stored at –80°C. All samples were blinded to avoid bias.

High-resolution melting analyses (HRMA) were performed to screen for mutations in the *NOTCH3* gene, using CFX Connect™ Real-Time System (Bio-Rad, USA). The primers for HRMA were designed using online Primer3Plus software based on the published genome sequence of the *NOTCH3* gene. The primers for exon 4 *NOTCH3* qPCR reactions, had the following sequences: 5'-AGTCTGGAGGGGAGGTAGTC-3' (forward 1) and 5'-CACCCGGCACTCATCCAC-3' (reverse 1), 5'-GATGGACGCTTCCTCTGCT-3' (forward 2) and 5'-CACCCCTCTGACTCTCCTGA-3' (reverse 2). Briefly, 15 ng of genomic DNA was used for qPCR with the intercalating dye EvaGreen (SsoFast™ EvaGreen® Supermix, Bio-Rad, USA), 250 nM of each primer. Next, the melting analysis was performed, and the data were analysed by Melting Analysis software (Bio-Rad, USA). The cycling conditions of HRMA will

be provided upon request. The results of HRMA were confirmed by Sanger sequencing in the forward and reverse directions using the 3130xl Genetic Analyzer (Applied Biosystems HITACHI, USA) in the independent unit. The readings were aligned to the human reference genome with BioEdit Software (Tom Hall Ibis Biosciences, Canada) separately by two investigators.

The HRMA of *MTHFR* C677T polymorphism using CFX Connect™ Real-Time System (Bio-Rad, USA) was performed according to the protocol published by Norambuena *et al*. [26]. The genotyping of *APOE* polymorphism was performed according to a modified mismatch primer method also on CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA) [28].

Biochemical analysis

The plasma Hcy concentration was analysed by High Performance Liquid Chromatography with Electrochemical Detection (HPLC/EC) (Dionex, Germany/ESA, USA) according to the methodology developed in our laboratory. In brief, 75 µl of water was used to dilute 150 µl of EDTA plasma. Subsequently, the mixtures were incubated for 15 min with 25 µl of 10% solution of tris(2-carboxyethyl)phosphine (TCEP) in water. Next, the proteins were denatured by the addition of 500 µl 1M $HClO_4$ and removed by centrifugation for 10 min at 10,000 RCF. The supernatant was transferred to fresh tubes and injected into HPLC/EC system equipped with pre-column and C18 column (250 mm × 4.6 mm × 5 µm, ThermoFisher Scientific, Germany) and eluted with aqueous 25 mM phosphate buffer, supplied with 15 mM sodium dodecyl sulfate and acetonitrile (17% vol). The buffer was adjusted to pH = 2.9 with orthophosphoric (V) acid. The chromatograms were obtained and analysed by Chromeleon software.

Microscopic analysis

Examination of the biopsy material from the skin and skeletal muscle was performed at the level of light and electron microscopy. The frozen tissue sections stained with HE were examined in light microscopy. Tissue samples for examination in electron microscopy were fixed in 2.5% glutaraldehyde with post-fixation in 2% osmium tetroxide and routinely processed into epoxy resin. Semi-thin sections stained with toluidine blue were examined in the light microscope to select the areas required. Ultrathin sections were counterstained with uranyl acetate, lead citrate and examined in the transmission electron microscope (Opton DPS 109).

Results

Clinical presentations

A 36-year-old female patient (II-6) was routinely admitted to the Department and Clinic of Neurology, Poznan University of Medical Sciences on 15 January 2018 in order to perform a diagnostic process of large, diffuse, multifocal, bilateral hyperintense lesions on head MRI performed in October 2017 due to severe headaches. The headaches had been occurring for 15 years with an increasing frequency. Their intensity was up to 10/10 in visual analogue scale (VAS). They were pulsating and localized in the left frontoparietal region. The headache was paroxysmal with 1-2 attacks per month lasting 2-3 days. There were not any triggering factors. Each MA attack was associated with nausea, vomiting, photophobia and phonophobia and preceded by aura manifesting as paresis as well as sensory dysfunction of the right upper limb and sometimes vision disturbances. A slight relief was brought by paracetamol and non-steroidal anti-inflammatory drugs (NSAIDs). The woman had not had any clinical symptoms of central nervous system (CNS) ischemic or haemorrhagic episode and epileptic seizure in the past. Her inmates had not noticed any signs of cognitive impairment, apathy or worrisome mood changes in the patient. The psychological examination showed no abnormalities. Similar hyperintense areas on head MRI and headaches occurred in her father (I-1) and two sisters (II-2, II-11). In addition, the proband (II-6) suffered from hypothyroidism. The neurological examination revealed only innate divergent strabismus of the right eye. The Doppler ultrasound examination of carotid and vertebral arteries was normal as well. The genetic examination found Y189C mutation in the *NOTCH3* gene. After identifying the causative mutations, we extended the genetic testing into the proband's family (Fig. 1). Among the twelve subjects studied (II-2, II-4, II-6, II-8, II-9, II-11, II-12, III-1, III-2, III-3, III-6, III-7), three family members with Y189C of *NOTCH3* mutation (II-2, II-6, II-11) had neurological symptoms and signs of CADASIL. The proband (II-6) had the microscopic analysis performed of skin-muscle biopsy which confirmed the presence of GOM.

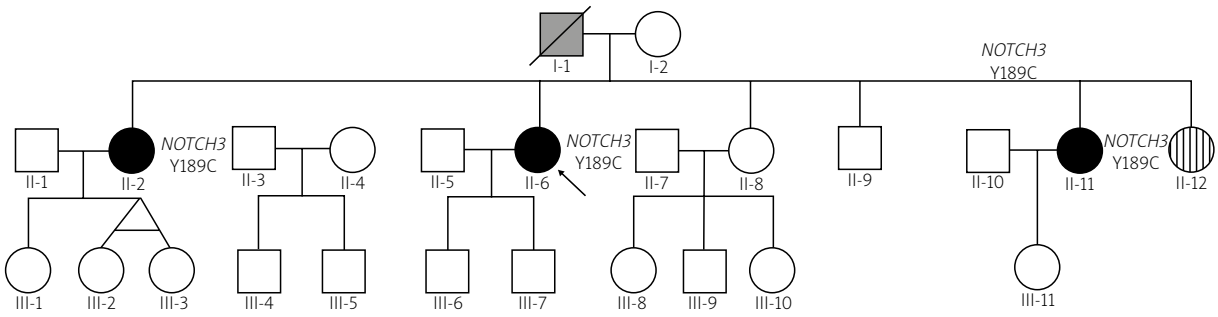

Fig. 1. The pedigree of the Polish family with Y189C *NOTCH3* mutation. Black – symptomatic CADASIL patients, grey – possible mutation carrier, striped – patient with Down syndrome.

Her 40-year-old sister (II-2) has been suffering from a headache for 20 years with intensity up to 9/10 in VAS. The headache started during pregnancy and became more severe after childbirth. It was pulsating and localized in the right frontoparietal region. The headache was paroxysmal with one attack per 2 months (earlier 3-4 attacks per month) lasting two days. There were no triggering factors. Each MA was associated with photophobia, phonophobia, osmophobia and preceded by aura manifesting by both visual (blurred vision, scotoma) and sensory (tingling, numbness) symptoms localized in the opposite side of a headache and lasting several minutes. The episodes of pain were sometimes accompanied by weakness of the limbs. A slight relief was brought by paracetamol and NSAIDs. The head MRI revealed numerous, scattered, hyperintense lesions. The psychological examination showed no abnormalities. The Doppler ultrasound examination of carotid and vertebral arteries were normal. In October 2018, she had the first transient ischemic attack (TIA).

The oldest daughter of patient II-2 (III-1) had few episodes of migraine without aura (MO). She is the only person among studied subjects carrying the heterozygous genotype of the common polymorphism of *NOTCH3*, the synonymous variant p.Ala202= (c.A606G, rs1043994). The MO was also present in her paternal grandmother.

Their 27-year-old sister (II-11) during genetic examination was pregnant and had no symptoms of CADASIL, thus the neuroimaging was postponed. However, she was admitted to the Department and Clinic of Neurology of the Poznan University of Medical Sciences from ER on 6 November 2018. She had three episodes of hemiparesis since the natural childbirth, which occurred about five weeks earlier. The episodes with the variable lateralization were associated with the visual symptoms (scotoma), global aphasia, weakness of the limbs and numbness lasting several hours. The symptoms were accompanied by osmophobia and headache localized in the opposite side. The intensity of headaches was up to 7/10 in VAS. The psychological examination found no abnormalities. The Doppler ultrasound examination of carotid and vertebral arteries were normal.

The clinical features of CADASIL of patients are summarized in Table I.

It is possible that CADASIL was inherited from the father (I-1), however the genetic testing was not performed. The first clinical symptom of CADASIL presented in the proband's father (I-1) was MA that started at the age of 43. The disease progression was rapid. He had numerous TIA and ischemic strokes. The strokes resulted in left-sided hemiparesis and encephalopathy. The patient died at the age of 51. His mother suffered from a severe MA, many ischemic episodes and decline in the cognitive function.

The proband's mother (I-2) is 62 years old and she has never had migraine and other symptoms of CADASIL. The siblings without the Y189C mutation of *NOTCH3* (II-4, II-8, II-9, II-12) do not suffer from MA, MO or stroke. The cognitive function impairment is present only in one daughter, woman with Down syndrome (II-12).

Genetic analysis

As mentioned before, the mutation in the exon 4 of the *NOTCH3* gene leading to the amino acid substitution Y189C was found in three symptomatic patients. This classical CADASIL mutation is localized in the EGF-like 4th domain of the NOTCH3 receptor and leads to the addition of a cysteine residue (Fig. 2).

Table I. Clinical features of the family members with the Y189C variant of *NOTCH3*

Parameter	II-2	II-6	II-11
Sex	Female	Female	Female
Age at study [years]	40	36	27
CADASIL-related symptoms	MA, TIA	MA	MA (3 attacks)
Age at migraine onset [years]	20, during pregnancy	21, stressful situation	27, after childbirth
Migraine attack duration	< 48 h	< 72 h	< 48 h
Migraine attack frequency	1/2 mths	1/mth	1/mth
MRI/CT	Ischemic changes	Ischemic changes	Ischemic changes
Doppler ultrasound	–	–	–
Psychiatric symptoms	–	–	–
Hcy [µmol/l]	11.1	12.5	5.2
Cholesterol [mg/dl]	–	167	243 (↑)
HDL [mg/dl]	–	64.0 (↑)	58.0 (↑)
LDL [mg/dl]	–	87.0	164.6 (↑)
MTHFR C677T genotype	CT	CC	CC
APOE genotype	E3/E3	E3/E3	E3/E3

MA – migraine with aura, TIA – transient ischemic attacks, CADASIL – cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, MRI – magnetic resonance imaging, CT – computed tomography, Hcy – homocysteine, MTHFR – methylenetetrahydrofolate reductase, APOE – apolipoprotein E

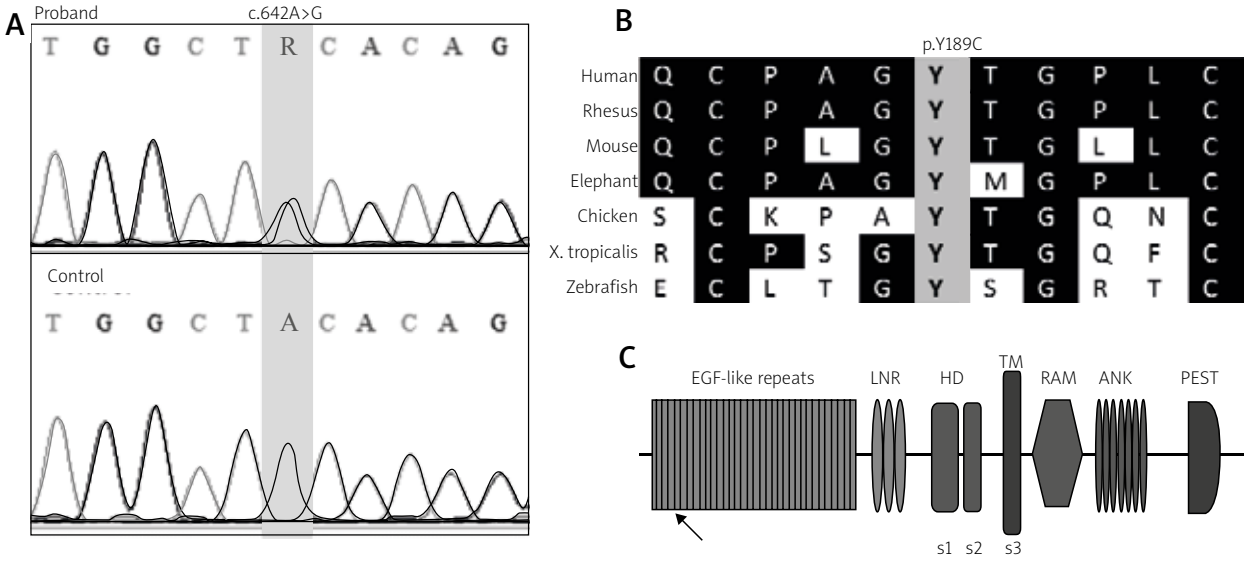

Fig. 2. Mutation Y189C of *NOTCH3*. **A**) Results of Sanger sequencing, **B**) Comparison of the NOTCH3 amino acid sequence around the mutation among different species, **C**) The structure of NOTCH3 protein, the arrow shows the position of the mutation. EGF – epidermal growth factor, LNR – Lin-12/NOTCH repeat, HD – heterodimerisation domain, TM – transmembrane, RAM – Rbp-associated molecule domain, ANK – ankyrin, PEST – peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T), s1-3 – site of protein cleavage.

The genotyping of the *APOE* polymorphism found the E3/E3 genotype in CADASIL patients with the Y189C *NOTCH3* mutation. Two sisters (II-6, II-11) were carriers of the CC genotype of *MTHFR*, while one (II-2) was a carrier of the CT genotype.

Neuroimaging

MRI of the proband (II-6) showed bilateral and multifocal fluid-attenuated inversion recovery (FLAIR) and T2-hyperintensities in the periventricular and deep white matter. A lot of lesions mainly affecting both anterior temporal lobes, frontal and parietal lobes, both symmetric external capsule, insulae and basal ganglia especially lenticular on the left side. There were symmetric hyperintense lesions in the pons. The subarachnoid cyst was found on the right side in the frontal lobe. Moreover, there was some widening subarachnoid space around the frontal lobes. All white matter lesions were smaller than 15 mm, most of them were oval or rounded. Cerebral ventricles were symmetric, not dilated. No microhaemorrhage lesions were found as well as no pathology in arteries and veins of the brain (Fig. 3B).

The proband's older sister (II-2) had four MRI examinations, in 2005, 2012, 2017 and 2018. They showed bilateral and multifocal FLAIR and T2-hyperintensities, mainly periventricular, which expand from 7 mm to 15 mm. Similar single and small changes were found in the corpus callosum and the pons. The MRI from 2018 showed the progression of the number of hyperintense lesions in the FLAIR and T2 additionally in the anterior parts of the temporal lobes up to 15 mm long. Hyperintense foci are located mainly perivascularly, at the expanded Virchow-Robin spaces (dPVS). The dPVS were widened of more than 3 mm. The numerous postischemic cavities were found on the left side in the centrum semiovale up to 11 mm. Some calcifications were localized on both sides in the deep nucleus and the cerebellum. There was no pathology in arteries of the brain (Fig. 3A). The patient had a CT scan with contrast in the 2002 year, at the age of 23, which showed small cortical atrophy of the brain, mainly in the frontal lobes. Amorphous calcifications were localized in the lenticular nucleus on both sides. Hypodense changes up to 10 mm in size on both sides of the external capsules correspond to areas of ischemia.

MRI of the proband's younger sister (II-11) showed bilateral and multifocal FLAIR and T2-hyperintensities mainly affecting frontal and parietal lobes up to 6 mm. No microhaemorrhages were found as well as no pathology in arteries and veins of the brain. A few symmetric calcifications were found in the deep nucleus on both sides in susceptibility weighted imaging (SWI) (Fig. 3C).

The CT scan without contrast showed hypodense changes up to 3 mm in size on right sides of the external capsules, which may correspond to small ischemic changes.

Microscopic analysis

Ultrastructural analysis of the material from skin-muscle biopsy was performed only in the proband (II-6) and revealed numerous GOM deposits in the vascular basal membrane (Fig. 4A), thickening of the vessel wall, and degenerative changes in endothelial cells (Fig. 4B), pericytes, and VSMC. In the

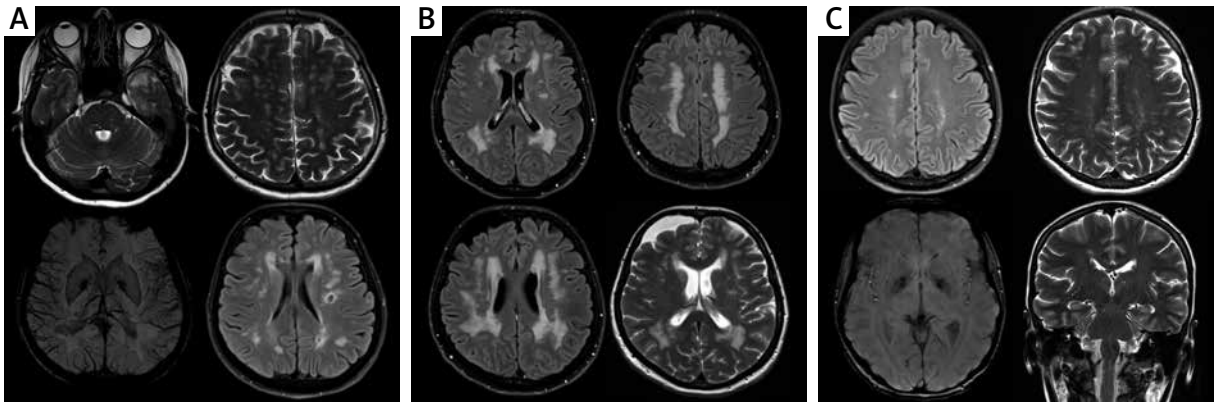

Fig. 3. MRI from 2018 of family members with the Y189C mutation in *NOTCH3*. **A**) Patient II-2, **B**) patient II-6, **C**) patient II-11.

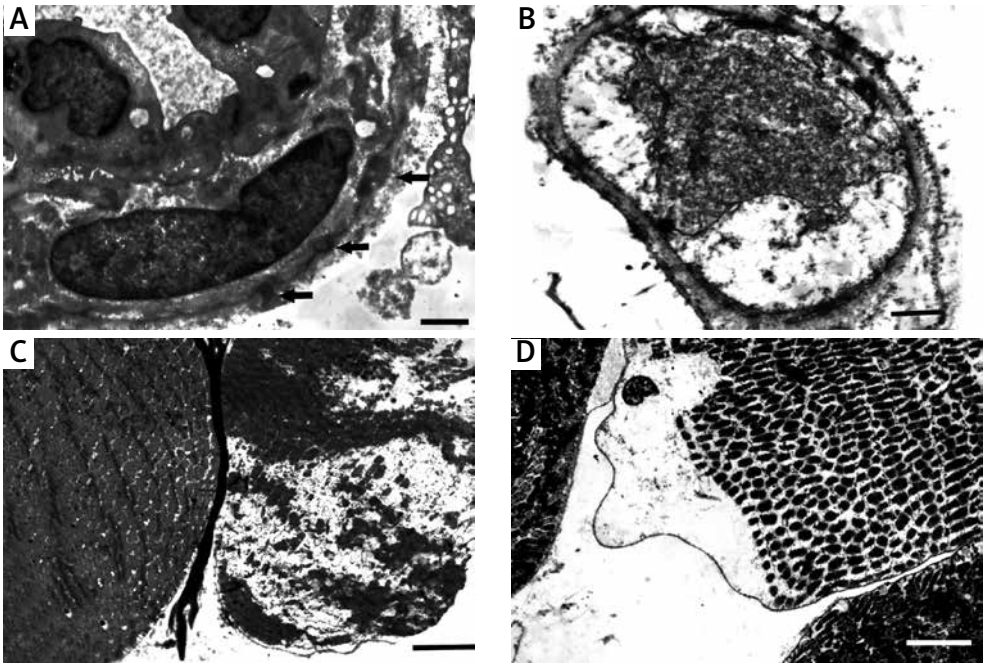

Fig. 4. Ultrastructural changes in the proband (II-6) biopsy material. **A**) GOM deposits in the media of the small skin artery; bar 2 µm. **B**) Swollen, pale endothelial cells in skeletal muscle arteriole, bar 1 µm. **C, D**) Rarefaction (C; bar 10 µm) and peripheral loss of myofibrils (D; bar 5 µm) in skeletal muscle fibres.

skeletal muscle sample, not only vascular changes but also structural abnormalities in muscle fibres were observed. A part of the muscle fibers showed the characteristic of ischemic damage rarefaction and/or peripheral loss of myofibrils (Fig. 4C,D).

Discussion

The present study is the first description of the Y189C *NOTCH3* mutation in Polish CADASIL patients. Moreover, it has never been correlated with the clinical symptoms of CADASIL. CADASIL is a rare monogenic neurological disease caused by a mutation in the *NOTCH3* gene. The typical mutations are localized in exons 2-24 encoding the EGF-like domains. As the majority of the mutation is present in exon 4 of *NOTCH3*, the genetic analysis should begin from screening this exon, and later should be extended to exons 2, 3, 5, 6 and 11 [20]. Mutations only in exons 3, 4, 6, 8 and 12 of *NOTCH3* have been identified in Polish CADASIL patients so far [2,7,21-23]. The mutations in exon 4 of the *NOTCH3* gene found in Polish patients include R133C [22], C185R and C212G [7]. The Y189C mutation in *NOTCH3*, analysed in our study, is also localized in exon 4, also in 4th EGF-like repeat of the protein. Rutten *et al*. [31] suggested that severity of CADASIL may be determined by the position of the *NOTCH3* pathogenic variant. They showed that patients with a mutation in EGF-like domain 1-6 were 12 years younger at stroke onset, had shorter survival time and larger brain lesions that patients with a mutation in EGF-like repeat 7-34. The phenotype of CADASIL associated with a mutation in EGF-like repeat 7-34 is milder with a broader spectrum ranging from attenuated CADASIL, late-onset small vessel disease, even to possibly non-penetrance.

According to our study, the Y189C mutation of *NOTCH3* was accompanied with CADASIL features such as MA, ischemic changes observed in MRI and CT, as well as characteristic degenerative changes in VSMC showed in microscopic analysis. The Y189C mutation of *NOTCH3* is a classic CADASIL mutation changing the number of cysteine residues from six to seven. There are two hypotheses explaining the role of mutations in *NOTCH3*. According to the first one, the misfolded NOTCH3 receptor gains new functions and leads to neurodegenerative changes in small vessels due to the increasing amount of proliferative myocytes, which leads to a chronic mechanical stress and chronic subcortical ischemic condition. It corresponds to the decrease in brain blood flow [6,8]. The second hypothesis classified

CADASIL as a proteinopathy in which misfolding of the NOTCH3 receptor results in aggregation of its extracellular domain and development of GOM deposits around the pericytes and VSMC. Aggregation of the protein is toxic to VSMC and pericytes and impairs the function of small vessels [15,27]. VSMC modifications are found early in the course of CADASIL and by affecting the meningeal and cortical vessels may contribute to the occurrence of MA [30]. However, the mechanism of MA in CADASIL is still unknown. The significant role in aura pathomechanism is played by the cortical spreading depression (CSD) [19]. The enhanced susceptibility to CSD is a result of the *NOTCH3* mutation as it was confirmed in a mouse model study [10]. The literature data indicate that the migraine features such as the age of onset, aura symptoms or attack frequency as well as other CADASIL symptoms may differ among family members with *NOTCH3* mutations [9,35]. All of them together suggest that heterogeneity of the CADASIL phenotype may be associated with modifying effects of factors other than causative *NOTCH3* mutations, e.g., hormonal status, Hcy level, *APOE* genotype as well as classic risk factors for stroke. Guey *et al*. [13] suggest that the hormonal status may influence the onset of MA in CADASIL as it facilitates the CSD. It may explain the occurrence of a migraine during pregnancy (II-2)/after childbirth (II-11) or its intensification after childbirth (II-6) in our patients with the Y189C mutation of *NOTCH3*. It was shown before that first symptoms of CADASIL, especially MA, may occur during pregnancy. Roine *et al*. [29] analysed the pregnancy history of 25 patients with the R133C *NOTCH3* mutation and found that 36% of females experienced neurological symptoms (e.g. hemiparesthesia, aphasia, hemiparesis, visual disorders) during gestation or puerperium as the first clinical manifestation of CADASIL. Fortunately, maternal CADASIL both in our study and a study of Roine *et al*. [29] did not influence the infant birth weight or Apgar points.

In the proband (II-6) the onset of MA was associated with a stressful situation, but in all sisters, MA started before the age of 30. According to a study performed on 119 CADASIL patients by Singhal *et al*. [34], younger age of MA onset in CADASIL patients may correlate with the elevated level of Hcy. However, hyperhomocysteinemia in CADASIL patients was rare (9%), while the hypercholesterolemia occurred in almost half of the patients (45%), and hypertension only in 20% of patients. In patients with the Y189C mutation in *NOTCH3*, the age of MA onset was not associated with the Hcy level; they had a Hcy level in the range of the reference values (5-15 µmol/l). One of the sisters (II-11) with this mutation had an elevated level of cholesterol, but she had only three MA attacks and no other CADASIL symptoms. It is difficult to say if the hypercholesterolemia may influence the course of the disease. However, the population-based study showed that adult MA patients have a higher risk profile of lipid metabolism than patients with MO. The increased cholesterol (≥ 240 mg/dl) was associated with MA [32], although it does not affect the features of MA, such as attack severity or frequency [35]. Among the CADASIL patients the higher incidence of atypical aura, e.g., motor deficit, long-lasting aura, was reported and may be useful in the diagnostic process [36]. The Y189C mutation of *NOTCH3* was also associated with a different spectrum of atypical aura symptoms, usually more severe than in a classic migraine without CADASIL, e.g., paresis or aphasia.

The MA frequency usually decreases with the CADASIL progression (stroke event). The supposed explanation involves changes at the cortex level involving myelin content or neuronal/microglial activity, but it needs further studies [13]. The ischemic changes both in CT and MRI were observed in all sisters carrying the Y189C mutation in the *NOTCH3* gene. The progression of ischemic changes in the older sister was noticed. In the MR, there are widening ischemic lesions in the subcortical parts of the temporal lobes with perivascular distribution. The widening of the dPVS was significant. In the oldest sister (II-2) they had a width of more than 3 mm. Furthermore, she has been suffering from CADASIL for 20 years and had the first TIA attack. The advanced ischemic changes and TIA may explain the decrease in frequency of migraine attacks in patient II-2. Patients with TIA or stroke in the disease history are more prone to subcortical dementia as a result of ischemic damage of the brain. Cognitive impairment in CADASIL starts with problems with information processing and executive functions, together with apathy and depression. During the disease progression, memory impairment occurs [1,4]. Our patients did not show any cognitive impairment or psychiatric disorders, and they were carriers of the population *APOE* E3/E3 genotype and had a normal level of analysed biothiol, which does not increase the risk of developing dementia in this way. On the other hand, our patients

with the Y189C *NOTCH3* mutation are young and cognitive symptoms may occur during progression of the disease as about 20-40% of CADASIL subjects suffer from psychiatric disorders, mostly depression or apathy [5].

The common polymorphism p.Ala202= of *NOTCH3* presented in subject III-1 was analysed previously in migraine or ischemic stroke patients. This variant probably is not a risk factor for migraine, as the polymorphic allele A was more common in healthy subjects than migraine individuals [33]. The three-generation family tree presented by Gallardo *et al*. [12] showed that p.Ala202= polymorphism was not efficient to cause CADASIL. The disease occurred only in patients carrying the classic *NOTCH3* mutation (in this case R90C), both with and without the p.Ala202= variant. According to the meta-analysis, this variant is not associated with ischemic stroke, both lacunar and atherothrombotic [18].

CADASIL is a rare disease and its diagnosis may be difficult in clinical practice. Moreover, the heterogenic manifestation of CADASIL symptoms makes the disease underdiagnosed because of difficulties in distinguishing with, e.g., MA, familial hemiplegic migraine or progressive ataxia. The *NOTCH3* mutation screening should be carried out in individuals with clinical features of CADASIL, even with the negative disease history [20,24].

The rapid development of molecular biology techniques may be useful in broadening the knowledge about the CADASIL pathomechanism and in designing the new personalized therapies based on genetic tests. As CADASIL is a monogenic disease, the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) is a promising technique in gene editing, both in designing the cell lines or animal models of disease and in gene therapy. Fernández-Susavila *et al*. [11] in 2018 generated and characterized the human iPSC isolated from a CADASIL patient carrying the *NOTCH3* mutation, which in the future may be used for the evaluation of the gene therapy in a CADASIL model.

Acknowledgments

We would like to thank members of the examined family for excellent cooperation.

Disclosure

The authors report no conflict of interest.

References

1. Brookes RL, Hollocks MJ, Tan RY, Morris RG, Markus HS. Brief Screening of vascular cognitive impairment in patients with cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy without dementia. Stroke 2016; 47: 2482-2487.
2. Buczek J, Błażejewska-Hyżorek B, Cudna A, Lusawa M, Lewandowska E, Kurkowska-Jastrzębska I, Członkowska A. Novel mutation of the NOTCH3 gene in a Polish family with CADASIL. Neurol Neurochir Pol 2016; 50: 262-264.
3. Chabriat H, Joutel A, Dichgans M, Tournier-Lasserve E, Bousser MG. CADASIL. Lancet Neurol 2009; 8: 643-653.
4. Di Donato I, Bianchi S, De Stefano N, Dichgans M, Dotti MT, Duering M, Jouvent E, Korczyn AD, Lesnik-Oberstein SA, Malandrini A, Markus HS, Pantoni L, Penco S, Rufa A, Sinanović O, Stojanov D, Federico A. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) as a model of small vessel disease: update on clinical, diagnostic, and management aspects. BMC Med 2017; 15: 41.
5. Dichgans M. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy: phenotypic and mutational spectrum. J Neurol Sci 2002; 203-204: 77-80.
6. Domenga V, Fardoux P, Lacombe P, Monet M, Maciazek J, Krebs LT, Klonjkowski B, Berrou E, Mericskay M, Li Z, Tournier-Lasserve E, Gridley T, Joutel A. Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. Genes Dev 2004; 18: 2730-2735.
7. Dziewulska D, Nycz E, Rajczewska-Oleszkiewicz C, Bojakowski J, Sulejczak D. Nuclear abnormalities in vascular myocytes in cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). Neuropathology 2018; 38: 601-608.
8. Dziewulska D. CADASIL – role of Notch 3 signaling system in pathomechanism of the disease. Aktualn Neurol 2011; 11: 237-243.
9. Dziewulska D. Mysteries of CADASIL – the contribution of neuropathology to understanding of the disease. Folia Neuropathol 2009; 47: 1-10.
10. Eikermann-Haerter K, Yuzawa I, Dilekoz E, Joutel A, Moskowitz MA, Ayata C. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy syndrome mutations increase susceptibility to spreading depression. Ann Neurol 2011; 69: 413-418.
11. Fernández-Susavila H, Mora C, Aramburu-Núñez M, Quintas-Rey R, Arias S, Collado M, López-Arias E, Sobrino T, Castillo J, Dell'Era P, Campos F. Generation and characterization of the human iPSC line IDISi001-A isolated from blood cells of a CADASIL patient carrying a NOTCH3 mutation. Stem Cell Res 2018; 28: 16-20.
12. Gallardo A, Latapiat V, Rivera A, Fonseca B, Roldan A, Sandoval P, Sánchez C, Matamala JM. NOTCH3 gene mutation in a Chilean cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy family. J Stroke Cerebrovasc Dis 2020; 29: 104530.
13. Guey S, Mawet J, Hervé D, Duering M, Godin O, Jouvent E, Opherk C, Alili N, Dichgans M, Chabriat H. Prevalence and char-

acteristics of migraine in CADASIL. Cephalalgia 2016; 36: 1038-1047.
14. Headache Classification Committee of the International Headache Society (IHS). The International Classification of Headache Disorders, 3rd ed. (beta version). Cephalalgia 2003; 33: 629-808.
15. Ihalainen S, Soliymani R, Iivanainen E, Mykkänen K, Sainio A, Pöyhönen M, Elenius K, Järveläinen H, Viitanen M, Kalimo H, Baumann M. Proteome analysis of cultivated vascular smooth muscle cells from a CADASIL patient. Mol Med 2007; 13: 305-314.
16. Joutel A, Andreux F, Gaulis S, Domenga V, Cecillon M, Battail N, Piga N, Chapon F, Godfrain C, Tournier-Lasserve E. The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients. J Clin Invest 2000; 105: 597-605.
17. Joutel A, Corpechot C, Ducros A, Vahedi K, Chabriat H, Mouton P, Alamowitch S, Domenga V, Cécillion M, Marechal E, Maciazek J, Vayssiere C, Cruaud C, Cabanis EA, Ruchoux MM, Weissenbach J, Bach JF, Bousser MG, Tournier-Lasserve E. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature 1996; 383: 707-710.
18. Keat Wei, Griffiths LR, Irene L, Kooi CW. Association of NOTCH3 gene polymorphisms with ischemic stroke and its subtypes: a meta-analysis. Medicina (Kaunas) 2019; 55: 351.
19. Kowalska M, Prendecki M, Kozubski W, Lianeri M, Dorszewska J. Molecular factors in migraine. Oncotarget 2016; 7: 50708-50718.
20. Kowalska M, Wize K, Wieczorek I, Kozubski W, Dorszewska J. Migraine and risk factors of vascular diseases. In: Pratap Sanchetee (ed.). Ischemic stroke of brain. IntechOpen, London 2018; 1-20.
21. Lewandowska E, Felczak P, Buczek J, Gramza K, Rafałowska J. Blood vessel ultrastructural picture in a CADASIL patient diagnosed at an advanced age. Folia Neuropathol 2014; 52: 443-451.
22. Lewandowska E, Leszczyńska A, Wierzba-Bobrowicz T, Skowrońska M, Mierzewska H, Pasennik E. Ultrastructural picture of blood vessels in muscle and skin biopsy in CADASIL. Folia Neuropathol 2006; 44: 265-273.
23. Lewandowska E, Wierzba-Bobrowicz T, Buczek J, Gromadzka G, Dziewulska D. CADASIL patient with extracellular calcium deposits. Folia Neuropathol 2013; 51: 302-311.
24. Maksemous N, Smith RA, Haupt LM, Griffiths LR. Targeted next generation sequencing identifies novel NOTCH3 gene mutations in CADASIL diagnostics patients. Hum Genomics 2016; 10: 38.
25. Mašek J and Andersson ER. The developmental biology of genetic Notch disorders. Development 2017; 144: 1743-1763.
26. Norambuena PA, Copeland JA, Krenková P, Stambergová A, Macek M Jr. Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the MTHFR gene. Clin Biochem 2009; 42: 1308-1316.
27. Opherk C, Duering M, Peters N, Karpinska A, Rosner S, Schneider E, Bader B, Giese A, Dichgans M. CADASIL mutations enhance spontaneous multimerization of NOTCH3. Hum Mol Genet 2009; 18: 2761-2767.
28. Prendecki M, Florczak-Wyspiańska J, Kowalska M, Lianer M, Kozubski W, Dorszewska J. Normal aging and dementia. In: Moretti DV (ed.). Update on dementia. InTech, Rijeka 2016; 251-272.
29. Roine S, Pöyhönen M, Timonen S, Tuisku S, Marttila R, Sulkava R, Kalimo H, Viitanen M. Neurologic symptoms are common during gestation and puerperium in CADASIL. Neurology 2005; 64: 1441-1443.
30. Ruchoux MM, Guerouaou D, Vandenhaute B, Pruvo JP, Vermersch P, Leys D. Systemic vascular smooth muscle cell impairment in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Acta Neuropathol (Berl) 1995; 89: 500-512.
31. Rutten JW, Van Eijsden BJ, Duering M, Jouvent E, Opherk C, Pantoni L, Federico A, Dichgans M, Markus HS, Chabriat H, Lesnik Oberstein SAJ. The effect of NOTCH3 pathogenic variant position on CADASIL disease severity: NOTCH3 EGFr 1-6 pathogenic variant are associated with a more severe phenotype and lower survival compared with EGFr 7-34 pathogenic variant. Genet Med 2019; 21: 676-682.
32. Scher AI, Terwindt GM, Picavet HSJ, Verschuren WM, Ferrari MD, Launer LJ. Cardiovascular risk factors and migraine: The GEM population-based study. Neurology 2005; 64: 614-620.
33. Schwaag S, Evers S, Schirmacher A, Stögbauer F, Ringelstein EB, Kuhlenbäumer G. Genetic variants of the NOTCH3 gene in migraine – a mutation analysis and association study. Cephalalgia 2006; 26: 158-161.
34. Singhal S, Bevan S, Barrick T, Rich P, Markus HS. The influence of genetic and cardiovascular risk factors on the CADASIL phenotype. Brain 2004; 127: 2031-2038.
35. Tana C, Santilli F, Martelletti P, di Vincenzo A, Cipollone F, Davì G, Giamberardino MA. Correlation between migraine severity and cholesterol levels. Pain Practice 2015; 15: 662-670.
36. Vahedi K, Chabriat H, Levy C, Joutel A, Tournier-Lasserve E, Bousser MG. Migraine with aura and brain magnetic resonance imaging abnormalities in patients with CADASIL. Arch Neurol 2004; 61: 1237-1240.